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The Acid Test: The Discovery of Two Pore Channels (TPCs) as NAADP-Gated Endolysosomal Ca^{2+} Release Channels

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Abstract

In this review we describe the background and implications of our recent discovery that Two pore channels (TPCs) comprise a novel class of calcium release channels gated by the intracellular messenger nicotinic acid adenine dinucleotide phosphate (NAADP). Their localization to the endolysosomal system highlights a new function for these organelles as targets for NAADP-mediated Ca^{2+} mobilization. In addition, we describe how TPCs may also trigger further Ca^{2+} release by coupling to the endoplasmic reticular stores through activation of IP_3 receptors and ryanodine receptors.

Keywords

-Inositol 1,4,5-trisphosphate-; Pancreatic beta cells; Endothelin; cholecystokinin; Ca2+ -induced Ca2 + release; Ion channel; Ca2+ mobilization; Ca2+ stores; Calcium signalling; Calcium transient

NAADP as an intracellular Ca^{2+} signalling messenger

NAADP is the most potent Ca^{2+} releasing molecule described, discharging Ca^{2+} from Ca^{2+} storage organelles to elicit cytoplasmic Ca^{2+} signals at picomolar to low nanomalor concentrations in sea urchin egg preparations (34). In some mammalian cells, Ca^{2+} responses are evoked by as low as 10 nM NAADP (4). Measurement of cell and tissue levels have shown that NAADP is synthesised in response to activation of various cell surface receptors and other stimuli, confirming NAADP's role as an intracellular messenger (18,53). In contrast to the other established Ca^{2+} mobilizing messengers, inositol 1,4,5-trisphosphate (IP_3) and cyclic ADP ribose (cADPR), that release Ca^{2+} from the endoplasmic reticulum (ER) by activating IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs), respectively, NAADP is enigmatic since in many cells it appears to act via a distinct Ca^{2+} release mechanism, which resides not on the ER but on acidic organelles (14).

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NAADP was discovered as a trace contaminant of commercially available NADP (35). This may not be too surprising since the structure of NAADP is very close to that of NADP, differing only by the substitution of nicotinic acid as the base instead of nicotinamide. This small change, however, has enormous biological effects since whilst NADP is inactive, NAADP is very potent at generating Ca^{2+} signals. This discovery was made by Lee and colleagues whilst studying calcium release mechanisms in homogenates prepared from sea urchin eggs (15). This preparation has done much to advance our understanding of novel Ca^{2+} signalling mechanisms including the first demonstration that cADPR mobilizes Ca^{2+} through the activation of RyRs (20,44), and much of the ground work in studying the mechanisms of NAADP action has been gleaned from this system before being widened to mammalian preparations.

NAADP targets acidic organelles

The initial report on the Ca^{2+} mobilizing action of the then unidentified NADP-related molecule implicated a different organellar target to the ER since it released Ca^{2+} from a partially separate subcellular fraction of sea urchin egg homogenate (15). Later, NAADP evoked Ca^{2+} release was found to persist in homogenates and intact eggs even when the ER Ca^{2+} pool had been abrogated by the SERCA pump inhibitor, thapsigargin. Another feature of sea urchin eggs is that when centrifuged they stratify, that is they change from spherical to a more oblong shape with different organelles migrating to different poles. Under these conditions, IP_3 and cADPR mobilize Ca^{2+} from the nuclear pole where the ER is, but NAADP releases Ca^{2+} predominantly from the opposite pole (36). Taken together, these studies favoured a target for NAADP in organelles distinct from the ER, the traditional site of action of Ca^{2+} mobilizing messengers.

A major new lead in the physiology of NAADP came from the finding that in sea urchin eggs the major target for NAADP was acidic organelles such as reserve granules, the urchin egg's equivalent of lysosomes (14). Stratification studies revealed that acidic stores stained with lysotracker localised to the non-ER pole exactly where NAADP was found to act as described above. Further fractionation of sea urchin egg homogenates showed that binding sites for [^{32}P]NAADP co-localized with lysosomal markers. The isolated NAADP-sensitive stores showed ATP-dependent Ca^{2+} uptake that was insensitive to thapsigargin, but abrogated by protonophores and bafilomycin A1, a vacuolar proton pump inhibitor, suggesting a role for a proton-dependent Ca^{2+} uptake mechanism. The lysosomotropic agent glycyl-L-phenylalanine 2-naphthylamide (GPN) selectively abolished NAADP-induced Ca^{2+} release in both homogenates and intact eggs whilst leaving IP_3- and cADPR-sensitive mechanisms unaffected.

NAADP-evoked Ca^{2+} release has now been shown to be widespread in mammalian cells, and even in plants (21). In most cases where investigated, including neurones, cardiac myocytes, smooth muscle, exocrine cells, and platelets, NAADP also appears to release Ca^{2+} from acidic stores rather than from the ER. The study of NAADP thus has highlighted acidic stores as important Ca^{2+} storage organelles that can be discharged by their own intracellular messenger. The role of Ca^{2+} storage in the endolysosomal system may be pivotal to endomembrane fusion and trafficking and for other physiological functions of these organelles (47,48). Recently, it has been proposed that reduced lysosomal Ca^{2+} storage is a primary defect in Niemann-Pick type C1 disease, a lysosomal storage disease, and this is associated with abolition of NAADP-induced Ca^{2+} release (37).

Given the smaller size, and perhaps the lower Ca^{2+} content, Ca^{2+} release from acidic stores is often smaller than that evoked from the larger reticular ER network. However, even the first report of NAADP-evoked Ca^{2+} release in a mammalian cell type proposed that NAADP-induced Ca^{2+} signals also trigger a subsequent, larger Ca^{2+} release from the ER through the Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms of IP Rs and RyRs (9). That Ca^{2+} release in

response to NAADP in turn triggers CICR through both IP_3Rs and RyRs has been extensively studied in sea urchin eggs (11,12) and demonstrated in a wide range of mammalian systems (2,4,[7,9],22,39). Indeed, tight junctions, likely less than 100 nm across, between the RyR-containing sarcoplasmic reticulum and a subpopulation of lysosomes have been observed in vascular smooth muscle and these may constitute a "trigger zone" for the initiation of propagating Ca^{2+} signals by NAADP (32,33). Thus, bafilomycin completely abolishes NAADP responses whilst thapsigargin and IP_3R and RyR blockage may only reduce the amplitude of the responses (7,22,[32,39],41,52). NAADP thus not only has a role in regulating endolysosomal luminal Ca^{2+} content and cytosolic Ca^{2+} signals arising from these organelles, but also a second role in coordinating ER-mediated Ca^{2+} release. Such interactions have often confounded experiments designed to elucidate the identity of the NAADP receptor, with some proposing that RyRs are a direct target (16). As we have stated previously, the identification of NAADP binding proteins are required to resolve such controversies (21).

Distinct pharmacology and properties of the NAADP-regulated Ca^{2+} release mechanism

The initial descriptions of NAADP-evoked Ca^{2+} release from sea urchin egg homogenates showed that NAADP likely activated a novel Ca^{2+} release mechanism distinct from IP_3Rs and RyRs. This was based on the individual ability of IP_3, cADPR and NAADP to induce homologous desensitization of their target mechanisms without affecting the responsiveness of the other two and regardless of the order of the additions of each messenger (35). Log_{10} concentration-response curves for NAADP-induced Ca^{2+} release are quite unlike the familiar sigmoid curves seen for either IP_3 or cADPR. In sea urchin eggs, subthreshold concentrations of NAADP for Ca^{2+} release can nevertheless fully desensitize NAADP receptors (24), whilst in mammalian cells the concentration-response curves are bell shaped, with micromolar concentrations of NAADP inducing desensitization in the absence of apparent Ca^{2+} release. This latter phenomenon has been exploited to identify receptor mechanisms coupled to NAADP-mediated Ca^{2+} signalling (9,18).

In another approach, pharmacological antagonism of IP_3Rs by heparin or RyRs by ryanodine, or the selective cADPR antagonist analogue 8-NH_2-cADPR, did not affect the ability of NAADP to evoke a Ca^{2+} release response (19). Whilst IP_3Rs and RyRs are also Ca^{2+}-activated channels and can propagate Ca^{2+} release as regenerative waves through CICR, this property is apparently not shared by the intrinsic NAADP-sensitive Ca^{2+} release mechanism either in egg homogenates (10,23) or in intact cells (11). As outlined above, rather NAADP evokes local Ca^{2+} signals that may only be propagated and globalized by subsequent recruitment of IP_3Rs and RyRs. In addition, NAADP-induced Ca^{2+} release is markedly less pH sensitive than IP_3Rs and RyRs (11) allowing the messenger to efficiently act at acidic stores, where large pH variation is inevitable because protons are co-released with Ca^{2+} (43).

Some pharmacological agents have been reported to selectively block NAADP-induced Ca^{2+} release without affecting either IP_3Rs or RyRs. These include voltage-gated calcium channel (VGCC) blockers such as dihydropyridines (25) and the store-operated channel and TRPC channel blocker SKF96365 (42). Recently, a potent and selective membrane permeant NAADP antagonist, NED19, has been described, which will be invaluable in helping dissect the contribution of NAADP during Ca^{2+} signalling (45). Taken together, these data support the hypothesis that NAADP activates a unique Ca^{2+} release mechanism involving channels that are perhaps more similar to VGCC or TRP families than the two established ER Ca^{2+} release channel families, the IP_3Rs and RyRs.

Identification of TPCs as NAADP receptors

The search for the molecular identity of NAADP receptors had been a bumpy ride to say the least. Initially, multi-step fractionations of sea urchin egg homogenates were attempted and a protein target that bound to [^{32}P]NAADP, but was substantially smaller than IP_3Rs and RyRs, was demonstrated (3). However, the NAADP-binding proteins were not identified at the molecular level. Since the demonstration that NAADP elicited Ca^{2+} release from lysosome-related acidic stores, lysosomal localized cation channels have been considered as candidates of NAADP receptors. For example, mucolipin 1, or TRPML1, was tested and failed to show [^{32}P]NAADP binding or an NAADP-evoked Ca^{2+} response in heterologous systems (49). Despite these findings, another group showed that a TRPML1 antibody blocked NAADP-evoked currents in planar lipid bilayers incorporated with lysosomal membranes prepared from rat liver and coronary arterial myocytes (55,56) and siRNA silencing of TRPML1 expression attenuated lysosome-associated Ca^{2+} release in the myocytes (55). However, other experimental data suggest that TRPML1 functions either as a proton channel or an iron release channel at the lysosomes (17,50).

In a recent study, we demonstrated that two-pore channels (TPCs or *TPCN*s for gene names) function as NAADP receptors (8). Based on primary sequences, TPCs are novel members of the superfamily of voltage-gated cation channels. Hydropathy analyses revealed that the full-length protein contains 12 putative transmembrane (TM) segments that are segregated into two homologous domains of 6 TM segments each with an intervening hydrophobic loop between the fifth and the sixth segments (Fig. 1). This 6-TM organization resembles that of all 6-TM voltage-gated cation channel family members including the four repeated domains found in the pore-forming subunits of voltage-gated Ca^{2+} and Na^{+} channels (54). The sequence homology of the TPC TM domains is also closer to voltage-gated Ca^{2+} and Na^{+} channels than to K^{+} channels. In fact among the single domain 6-TM channels, Catspers and some TRP channels are more closely related to TPCs than K_v channels are, but nevertheless their homologies to TPCs are slightly lower than that of Ca_v and Na_v channels. This has led to the hypothesis that TPCs may represent evolutional intermediates of the two round duplications of single 6-TM domain channels that gave rise to the four 6-TM domain Ca_v and Na_v channels (1). The fact that related TPCs are widely expressed in plants suggests that this is an ancient ion channel family that had been in existence before the divergence of plants and animals. On the other hand, *TPCN* genes are completely missing in some model animal species, such as flies and nematodes, indicating that these channels may not be essential for survival.

With the report of the first mammalian TPC (TPC1) sequence some years ago (28), so was the name given but this should not be confused with two-pore potassium channels, which have two repeats of a 2-TM architecture. However, functional data had been completely lacking, at least in animal cells, for this unique group of ion channels that apparently have an important position in the evolution of voltage-gated cation channels. By contrast, active research on plant cells revealed that plant TPC1, which is not equivalent to animal TPC1 but rather an equally distant homologue of all animal TPCs, forms a Ca^{2+} release channel in intracellular vacuoles (46). Indeed, even before the work on the plant TPC1 was reported, we had noticed that human TPC1 and TPC2 expressed in HEK293 cells were not expressed on the plasma membrane but rather on membranes of endosomes and lysosomes. Given that NAADP acts at acidic endolysosomal stores (14) and its action is inhibited by Ca^{2+} channel antagonists (25), the specific subcellular locations and the homology to Ca_v and TRP channels prompted us to examine whether TPCs form NAADP receptors.

Because of its prominent localization on lysosomal membranes, we focused initially on TPC2 and obtained several lines of evidence that TPC2 is involved in NAADP binding and NAADP-mediated Ca^{2+} release from acidic stores (8). First, NAADP binds to membranes from cells

over-expressing TPC2 with a high affinity of 5-10 nM. Second, NAADP, at concentrations as low as 10 nM, elicited intracellular Ca^{2+} concentration increases in HEK293 cells that overexpress TPC2. Interestingly, the rise in intracellular Ca^{2+} exhibited two phases, an initial pacemaker phase with low to moderate Ca^{2+} transients akin to a "Ca^{2+} burst" (4), followed by a secondary rapid and much larger Ca^{2+} increase. The initial phase represents lysosomal Ca^{2+} release evoked by NAADP, as it was unaffected by blocking ER Ca^{2+} release with thapsigargin, heparin, or ryanodine, or all of these together, but completely abolished after treating cells with bafilomycin. The secondary phase, however, was eliminated by thapsigargin or heparin, or both of these together with ryanodine, and therefore represents globalization of the Ca^{2+} signal through CICR, resulting, originally, from the NAADP-induced Ca^{2+} release from acidic stores. This biphasic phenomenon and the dependence of the secondary phase on the ER stores are entirely consistent with NAADP-elicited Ca^{2+} responses in native cells as described above. Interestingly, the wild type HEK293 cells lacked the biphasic response and exhibited only small and short-lived Ca^{2+} transients upon stimulation by higher concentrations of NAADP, suggesting that the relatively low endogenous level of TPC expression in this cell type does not favour globalization of the NAADP-evoked Ca^{2+} signal and / or that lysosomes form "loose junctions" with the ER in HEK293 cells and thereby fail to adequately mediate, via endogenous TPC2, sufficient Ca^{2+} release to breach the threshold for CICR via IP_3Rs. Third, shRNA specifically targeted against TPC2 abolished NAADP-evoked Ca^{2+} signals in HEK293 cells over-expressing TPC2 and the native NAADP response in a human hepatoblastoma cell line. Finally, NAADP-evoked Ca^{2+} signals activated non-selective cation currents in pancreatic b cells prepared from wide type mice, but no such current was detected in cells prepared from *Tpc2* knockout mice. Taken together, these data strongly support a role for TPC2 in NAADP-evoked Ca^{2+} release in mammalian cells.

We further noticed that although overexpression of TPC1 did not result in globalization of the Ca^{2+} signal in response to 10 nM NAADP, the localized Ca^{2+} transients did become more pronounced at this low ligand concentration than in wild type cells. This, perhaps, reflects the more discrete intracellular distribution of TPC1 than TPC2, the lower Ca^{2+} content in the TPC1-expressing endosomes than lysosomes and or the fact that endosomal TPC1 does not form true junctions with the ER in HEK293 cells and therefore fails to elicit CICR via IP_3Rs. Nevertheless this finding does suggest that TPC1 may also act as an NAADP receptor. Therefore, the TPCs represent a family of endolysosomal cation channels targeted by NAADP for regulated Ca^{2+} release from acidic stores. Ca^{2+} release from this dynamic and mobile source contributes to the diversity and versatility of Ca^{2+} signalling not only because of its unique origin, but also because of its ability to couple to the ER Ca^{2+} release under certain but not all conditions (Fig. 2).

Physiology of TPC-mediated calcium signalling

There is still much to learn about TPC-mediated Ca^{2+} signalling. The NAADP-induced Ca^{2+} responses in sea urchin and starfish eggs are pivotal events at fertilization (13,42). In mammalian systems, this ligand appears to play key roles in digestive enzyme and fluid secretion of pancreatic acinar cells (52), glucose-induced insulin secretion in β cells (40), smooth muscle contraction in the vasculature (4,32), T lymphocyte activation (2), neurotransmitter release and neurite outgrowth (5,6), and platelet activation (38). In some cases NAADP evokes specific Ca^{2+} responses distinct from either IP_3 or cADPR such as the cortical flash at marine invertebrate egg fertilization (13,42), and differentiation of PC12 cells (5). In addition, NAADP modulates luminal pH of its target organelles suggesting another distinct signalling event evoked by this messenger (43). Notwithstanding, the study of NAADP signalling had been greatly hindered by the lack of knowledge about its molecular target(s) and lack of specific blockers. Now two recent reports have revealed not only a potent and specific NAADP antagonist (45), but also the molecular identity of the NAADP receptor (8).

It is expected that these findings will greatly accelerate functional characterization of NAADP-induced Ca^{2+} signalling in many different cell types and biological systems (Fig. 2).

Human genetic studies have revealed some physiological and pathophysiological functions that may be attributed to TPC2. The polymorphic variants of the *TPCN*2 gene at two locations are associated with hair colour in Northern Europeans (51), indicative of an important role for this channel in pigmentation. This would be consistent with the lysosomal function of TPC2 in melanosomes, lysosomal-related organelles of the melanocytes, which produce and release melanin to keratinocytes for pigmentation. The *TPCN*2 locus is also within the chromosomal region that is linked to autosomal recessive nonsyndromic hearing impairment in at least five families (29,31), and for one of them, the *TPCN2* gene has been sequenced to show five exonic and four intronic variants (29). Given that progressive hearing loss has been linked to several lysosomal storage diseases (30), it is possible that dysfunction of the lysosomal Ca^{2+} release channel, TPC2, contributes to this disease. Furthermore, *TPCN2* is one of three genes within the 11q13 amplicon core found in many human oral cancers that are frequently overexpressed in nonamplified primary tumors, indicating that it may contribute to growth or metastatic potential of cancer cells (27). Recent studies have linked alteration of Ca^{2+} signalling in apoptosis and autophagy of cancer cells (26). Autophagy is a physiological process invoked by cellular metabolic stress that is known to involve lysosome-mediated proteolysis. However, there has been very limited study of the role of lysosomal-mediated Ca^{2+} signalling in regulation of autophagy and apoptosis. Understanding the functional relationship between NAADP and TPC2-mediated Ca^{2+} release could provide an attractive therapeutic strategy for modulating the synergistic relationship between apoptosis and autophagy in cancer cells.

Conclusions

The discovery of TPCs as NAADP-gated Ca^{2+} release channels in the endolysosomal membranes opens many new directions for investigating the role of Ca^{2+} signalling in human health and diseases. Continued investigation in this area should offer great insights into the roles of the local NAADP signalling through endolysosomes and its coupling to global Ca^{2+} events through communication with the ER Ca^{2+} store, as well as to other systems, such as plasma membrane channels and Ca^{2+}-dependent enzymes (Fig. 2). Therefore, this diverse and versatile signalling capability likely contributes to many physiological processes. Moreover, although our current study demonstrates unequivocally that TPCs function as the NAADP-regulated Ca^{2+} release channels from acidic organelles, we cannot rule out the possibility that other proteins tightly associate with these channels and modulate their activities. It may also be too early to rule out the direct involvement of other candidate channels, e.g. RyRs and TRPML1, in NAADP-evoked Ca^{2+} mobilization because this will require additional experimentation under specific conditions in appropriate cell types.

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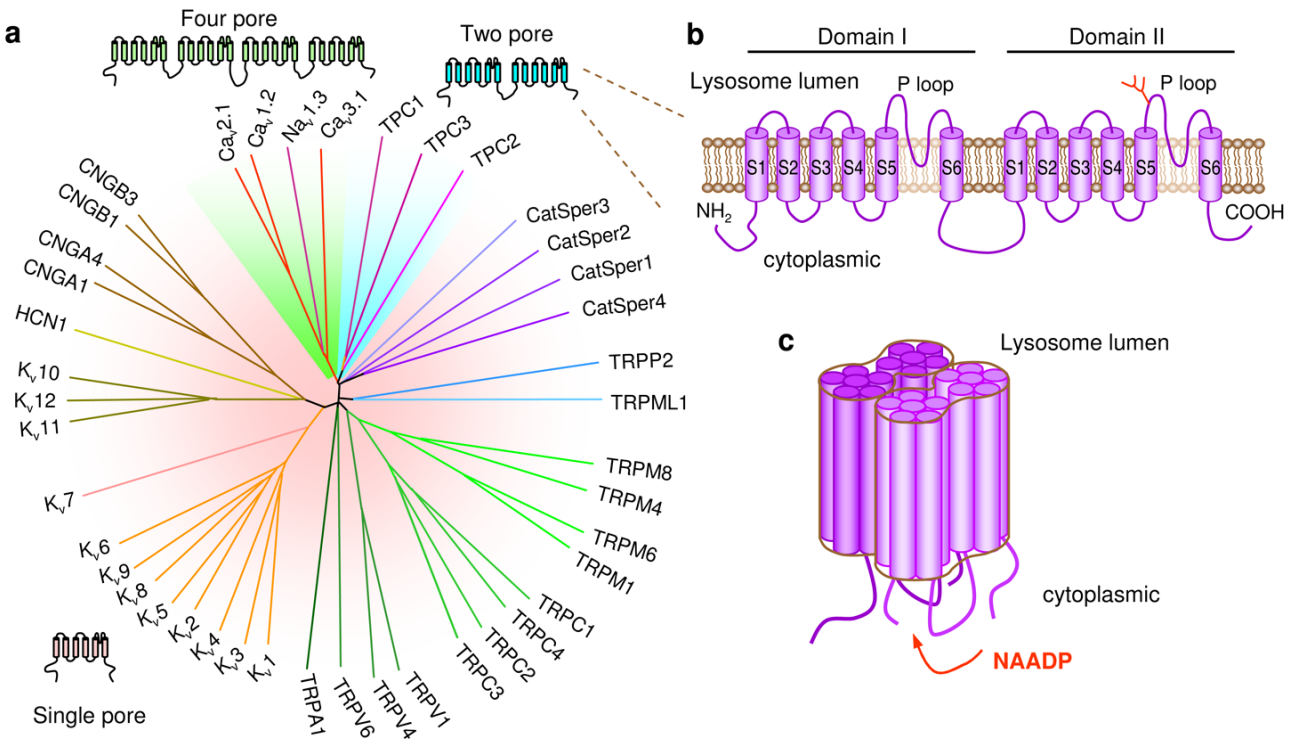

Fig. 1. Two-pore channels: relationships with other voltage-gated channels and structure organization

a. A phylogenetic tree generated by ClustalW (http://align.genome.jp) and plotted using Neighbour-Joining algorithm for TPCs and selected members of the superfamily of voltage-gated channels. The second transmembrane (TM) domain of TPCs and the fourth TM domain of Ca_V and Na_V were aligned with the TM domains (including all six TM segments and the pore-loop) of representative K_V, CNG, TRP, and CatSper channels. **b**. A single TPC subunit contains 12 putative TM segments that can be divided into two 6-TM homologous domains, each containing also a pore-loop (P-loop). Based on orientation of voltage-gated channels, the amino and carboxyl termini, as well as the large loop in between the two domains, are on the cytoplasmic side of the endolysosomal membranes. Red branches indicate a putative glycosylation site in human TPC2. **c**. 3-D projection model of an assembled TPC channel, which is most likely a dimer.

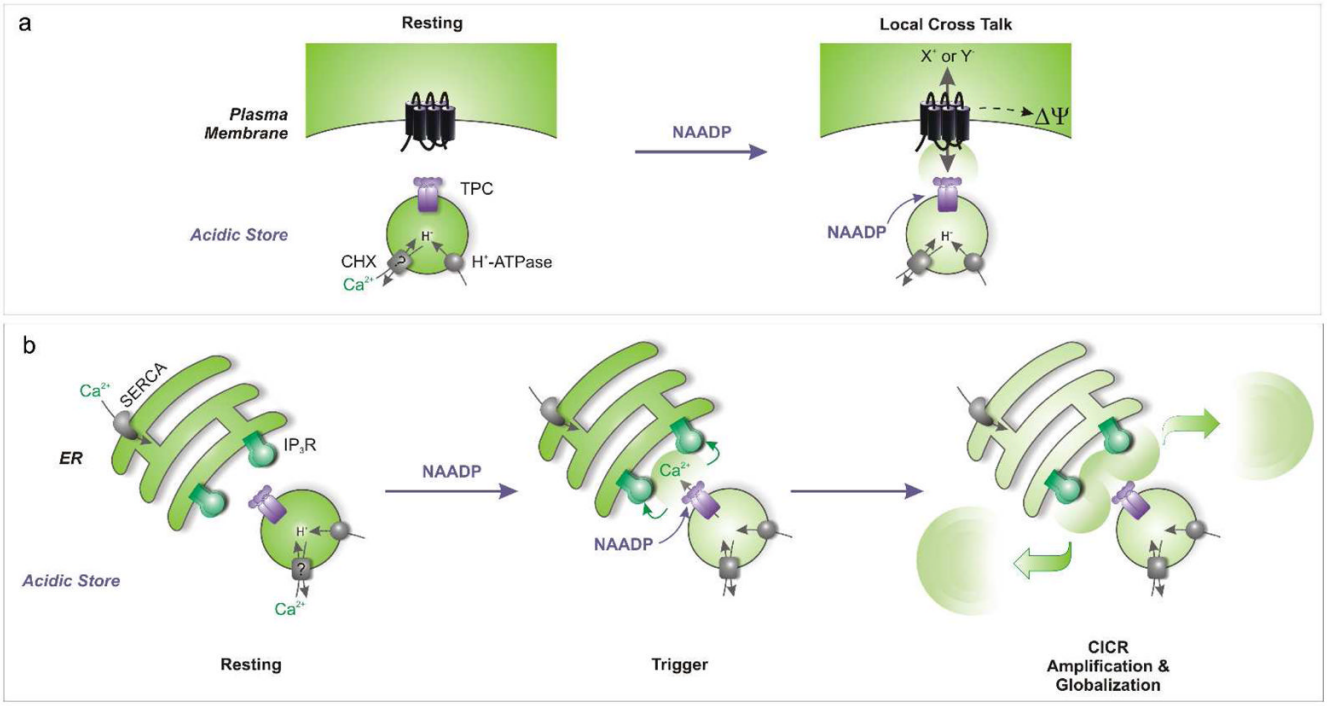

Fig. 2. NAADP regulation of TPCs

Model outlining how NAADP-regulated TPCs expressed at acidic Ca^{2+} stores might modulate the activity of closely apposed Ca^{2+}-sensitive channels in, **a** the plasma membrane or **b,** endoplasmic reticulum via local Ca^{2+} (represented by the green fill).

a, Ca^{2+}-sensitive ion channels in the plasma membrane are arbitrarily depicted with six transmembrane domains and might be cation- or anion-selective, inward or outward currents (double-headed arrow). NAADP-evoked Ca^{2+} release from sub-plasmalemmal acidic stores produces a local Ca^{2+} signal that activates plasma membrane channels (7,8,9). In turn, this may drive changes in the plasma membrane potential.

b, A two-pool model of Ca^{2+} spiking where a highly localized trigger Ca^{2+} released via TPCs recruits CICR channels on the ER (8).

Abbreviations: CHX, Ca^{2+}/H+ exchanger; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; IP3R, IP3 receptor; ER, endoplasmic reticulum; CICR, Ca^{2+}-induced Ca^{2+} release; X+, cations; Y−, anions; Ψ, membrane potential. Drawn by Dr Anthony Morgan.